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Effect on Alpha-Amylase Production by Employing Polyethylene Glycol at Different Concentrations in Medium

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ABSTRACT

Amylases are extracellular enzymes of great industrial interest. Present study was planned with the objectives- comparison and screening of best α-amylase producer bacteria, effect of PEG 4000 in different concentrations on α-amylase production and, recovery of α-amylase from crude extract, concentrate and ultra-filtrate. It was found that *Bacillus amyloliquefaceins* had 39.5 mm and *Bacillus subtilis* 29.5 mm diameter of zone of clearance on starch agar medium, while the isolate from soil showed much lesser (5.5 mm) zone of clearance. Therefore, *Bacillus amyloliquefaceins* strain was best α-amylase producer than others. Medium with 7 and 2% PEG concentration showed 30 times higher alpha amylase activity than medium without PEG. Furthermore lower concentration of PEG 2% showed promising results as compared to PEG 7%. The highest concentration of α-amylase (2690 U mL^{-1} of cf) was found in sample concentrate, which is nearly ten times higher than the original sample (260 U mL^{-1}). Theoretically ultra filtrate should not have any amylase activity but in our result it was 89 U mL^{-1}. Present study suggests that *B. amyloliquefaceins* is the best bacterial strain to produce maximum quantity of α-amylase in the medium containing PEG 2%.

Key words: α-amylase, PEG, *B. amyloliquefaceins*, ultrafiltrate

INTRODUCTION

Alpha-Amylases (α-1,4-glucan-4-glucanohydrolase) are starch-degrading enzymes that catalyze the hydrolysis of internal α-1,4-O-glycosidic bonds in polysaccharides with the retention of α-anomeric configuration in the products or decomposing them in glucose (Sivaramakrishnan *et al*., 2006). Starch is a major source of carbohydrates in the nature, being an abundant carbon source. Every starch polysaccharide molecule possesses one reducing terminal, able to reduce dinitrosalicylic acid; therefore, measurement of reducing sugars is an useful method to determine the molar concentration of starch molecules in solution. Amylases are extracellular

enzymes of great industrial interest. α-amylases are the enzymes first to be commercially produced and marketed. Amylases possess important applications in the production of syrup with high glucose content, sweetener manufacture, nourishing, detergents and ethanol. Other applications include food, textile and paper industries (Pandey *et al*., 2000). The food and beverage sectors utilize 90% of the carbohydrases produced. Due to the increasing demand for these enzymes in various industries, there is enormous interest in developing enzymes with better properties such as raw starch degrading amylases suitable for industrial applications and their cost effective production techniques (Burhan *et al*., 2003). To meet the demand of industries, low-cost medium is required for the production of α-amylase. Both solid Substrate Fermentation (SSF) and submerged fermentation (SmF) could be used for the production of amylases, although traditionally these have been obtained from submerged cultures because of ease of handling and greater control of environmental factors such as temperature and pH. The term fermentation defined biochemically is the energy yielding process in which organic molecules serves as electron donor as well as acceptor both. The annual sale of α-amylases in global market is estimated to be $11 million (Kilara and Desai, 2002). In spite of the wide distribution of amylases, microbial sources, namely fungal and bacterial amylases, are used for the industrial production due to advantages such as cost effectiveness, consistency, less time and space required for production and ease of process modification and optimization. Bacterial α-amylase, however, is preferred over fungal amylase due to several characteristic advantages that it offers. Among bacteria, *Bacillus* sp., is widely used for thermostable α-amylase production to meet industrial needs. Keeping all these facts in the mind, the present study was planned to study the effect of deferent concentrations of polyethylene glycol (PEG) on α-amylase production with low cost of production. Study was planned with the following detail objectives (1) Isolation, comparison and screening of best α-amylase producer bacteria, (2) Effect of PEG 4000 in different concentrations on α-amylase production and (3) Recovery of α-amylase from crude extract, concentrate and ultra-filtrate.

MATERIALS AND METHODS

Reagents and media: Polyethylene glycol (PEG)-4000 was obtained from Sigma (St. Louis, MO, USA) and used as 2 and 7% concentration in the production medium. All other reagents were of analytical grade.

Preparation of media was carried out by measurement of weight on electronic balance FX-3200 (National, Japan). Nutrient agar was used as primary media for isolation of organism. The composition of basal media was as (g L^{-1}): 5 $(NH_4)_2SO_4$; 9 K_2HPO_4; 3 KH_2PO_4; 1 Sodium citrate; 0.1 $CaCl_2$ $2H_2O$; 0.14 $FeCl_3$ $6H_2O$; 0.01 $FeSO_4$ $7H_2O$; 0.5 $MgSO_4$ $7H_2O$; 0.01 $MnSO_4$ H_2O; 0.001 $ZnSO_4$ $7H_2O$ and 1 yeast extract. Starch agar media was prepared by adding 1% starch, 0.1% agar and 1.5% yeast in basal media, mixed thoroughly and sterilized. Finally prepared starch agar plates and slants in McCortney bottles. Prepared the 50 mL production media by taking 50 mL basal media and add 0.1% yeast extract and 1% tapioca (Cassava) starch (as production starch) in four different flasks for the time course of four days. All media were sterilized in autoclave at 15 lb/in^2 pressure (121°C) for 15-30 min.

Source and selection of microorganism: The present study was conducted during the June, 2006. Two bacteria namely *Bacillus subtilis* and *Bacillus amyloliquefaceins* were obtained from the culture collection of the University of Western Sydney, Hawkesbury campus, Sydney, Australia. While isolate was obtained from soil by isolation and screening procedures as described.

Soil sample was used as source for micro organism (isolate), approximately 1 g of sample was taken and mixed with 100 mL of basal medium which also contains 1% starch and 0.1% yeast extract (nitrogen source) and was shaken for 24 h in rotary shaker. The isolate was identified by morphological characteristic, Gram's staining and Biolog method. Compare the isolated organism with best given standard strains (e.g., *Bacillus subtilis* and *Bacillus amyloliquefaceins*). All three samples (cultures) were then poured on the starch agar plates and were incubated for overnight to follow up with a replica plate. The next day, they were screened for best α-amylase producer by semi quantitative estimation method. Spot plate technique was carried out (Laboratory Manual, 2006) and measures the Zone of Clearance (ZOC) on starch agar plate. Zone of clearance method (Laboratory Manual, 2006) is used for the semi quantitative estimation. In brief, a small drop of 0.1 mL of cultured micro-organism was placed on the surface of the starch agar plates and incubated for 48 h at 37°C and after incubation, zone of clearance was observed. Zone of clearance was checked by placing iodine solution on the plate. The cultured micro-organisms that produced the maximum zone of clearance were the best amylase producer. Cultures were maintained properly throughout the experiment once in a fortnight by using starch agar plates and slants. The cultures were streaked in to new starch agar plates and slants by selecting from a healthy colony of plates that were free of contamination and after overnight incubation placed in refrigerator (4°C) to prevent the further growth of the organisms.

Inoculum preparation: Inoculation media was prepared by adding 0.1% yeast extract and 0.5% soluble starch in 50 mL basal media and sterilized in autoclave at 15 lb in^{-2} pressure (121°C) for 15-30 min. After cooling at room temperature, bacterial culture was aseptically transferred to each flask. The inoculum size was 5% (v/v).

Fermentation technique and amylase production: Amylase production was done by employing batch fermentation technique (Stanbury *et al*., 2000). Fermentation was carried out by two ways- Shake flask and Bioreactor. For fermentation with Shake flask, took 50 mL production media in four 250 mL conical flask and sterilized in autoclave. After sterilization production media was cool down and inoculated each flask with the inoculum size 5% (v/v) and placed in stationary incubator at 37°C. The time courses were carried out for a period of four days and samples were collected at interval of 24 h. Analyses of the samples were carried out by first thawed at room temperature and centrifuged at 8000xg at 4°C for 10 minutes. Supernatant was collected and used for analysis of α-amylase, reducing sugar and total protein. Fermentation with Bioreactor, 800 mL production media was took in 1 L Braun Biolab fermentor vessel. Other steps were same as Shake flask method.

Fermentation time courses: Most of the time courses were carried out in McCartney bottles over a period of four days. Alpha-amylase, reducing sugar (Cassava starch) and total protein were measured at 24 h interval. The fermentation was executed in four time courses:

- **Time course 1:** Bioreactor 1 L with standard condition (Temperature 37°C, pH 7.0, Air volume 1100 cm^3 min^{-1}, Stirrer speed 2.5×0^2 rpm)
- **Time course 2:** Shake flask (250 mL)
- **Time course 3:** Shake flask (250 mL)-2% PEG is added to production medium
- **Time course 4:** Shake flask (250 mL)-7% PEG is added to production medium

In the first two time courses (1 and 2) no primer (PEG) was added, while in last two time courses (3 and 4) PEG was added as 2 and 7%, respectively to study the effect of PEG in different concentrations on α-amylase production.

Isolation and extraction of α-amylase: In the fermentation broth of Bioreactor the enzyme is available in crude form along with other products such as proteins, nucleic acid etc. but only α-amylase is the required product so for the isolation and extraction of the α-amylase downstream processing was carried out. Extraction was done by ultra filtration method (Wilson and Ingledew, 1982). The filtrate and retentate were assayed to check the enzyme concentration.

A-amylase assay: The α-Amylase assay was done as per the method of Wilson and Ingledew (1982). The substrate consisted of 0.2% soluble starch dissolved in boiling 0.05 M KH_2PO_4-NaOH buffer (pH 6.0) and cooled to 40°C. The iodine reagent was prepared fresh by diluting 1 mL of stock solution (0.5% I_2 in 5.0% KI) into 500 mL of deionized water containing 5 mL of 5N HCI. For the assay, 1.0 mL of enzyme solution was placed in a test tube (16 by 150 mm) and warmed to 40°C in a water bath. At 10 min after the addition of 2.0 mL of starch substrate, the reaction was stopped by taking a 0.2 mL sample and adding it to 5.0 mL of iodine reagent. The absorbance at 620 nm was measured against a blank (0.2 mL of water in 5 mL of iodine reagent). The substrate control used 1.0 mL of buffer in place of the enzyme. Amylase activity was calculated from the absorbance by using the following equation:

$$\alpha\text{-amylase units per milliliter} = [(\text{control-test})/\text{control}] \times 40D$$

where, D is the enzyme dilution factor and 40 represents the 4.0 mg of starch present in the reaction tube times 10.

One unit of a-amylase is defined as the amount of enzyme that will hydrolyze 0.1 mg of starch in 10 min at 40°C when 4.0 mg of starch is present. Activities which resulted in absorbances of <0.125 after 10 min required dilution to give linear reactions over the 10 min period.

Determination of reducing sugar and protein: Reducing sugar was estimated by Dinitrosalicylic Acid (DNS) Method (Miller *et al.*, 1960). Protein was determined by the method of Lowry *et al.* (1951), using Bovine Serum Albumin (BSA) to prepare the standard curve.

RESULTS

In the present study the first step was to isolate the desired microorganism that produces more amount of α-amylase. Zone of clearance was checked by placing iodine solution on the plate. No colour is produced in the zone of clearance as the starch will be utilized by the amylolytic organisms. The species of isolate was not confirmed even by Biolog method. It was found that *Bacillus amyloliquefaceins* had 39.5 mm and *Bacillus subtilis* 29.5 mm diameter of zone of clearance, while the isolate from soil showed much lesser (5.5 mm) zone of clearance. Therefore, we decided to choose *Bacillus amyloliquefaceins* to fulfill our aim of increase amylase production. So, in subsequent time courses we used *Bacillus amyloliquefaceins* for increased production of α-amylase in the medium containing PEG 4000. The present findings are in agreement with the report of Sivaramakrishnan *et al.* (2006).

Table 1: Time-courses of *Bacillus amyloliquefaciens* on 1% Cassava starch in 1 L Braun bioreactor with fermentation conditions: Temp. 37°C, pH 7.0, Air volume 1100 cm^3 min^{-1}, Stirrer speed 2.5×10^2 rpm

Time-days	pH	Amylase activity U mL^{-1} of cf	Protein µg mL^{-1} of cf	Reducing sugar µg mL^{-1} of cf
0	6.80	0.52	83.0	790
1	6.49	3.11×10^2	336.9	690
2	6.63	4.0×10^2	310.4	150
3	6.63	2.6×10^2	324.4	70

cf: Culture fluid

Table 2: Time course of isolate grown on Cassava starch in shake flasks under standard conditions: Temperature 37°C, inoculum size 5% (v/v), substrate concentration 1% (w/v) and aerobic conditions

Time-days	Amylase activity U mL^{-1} of cf	Protein µg mL^{-1} of cf	Reducing sugar µg mL^{-1} of cf
0	1.5	54	-
1	5.1	188	-
2	3.4	174	-
3	2.9	96	-

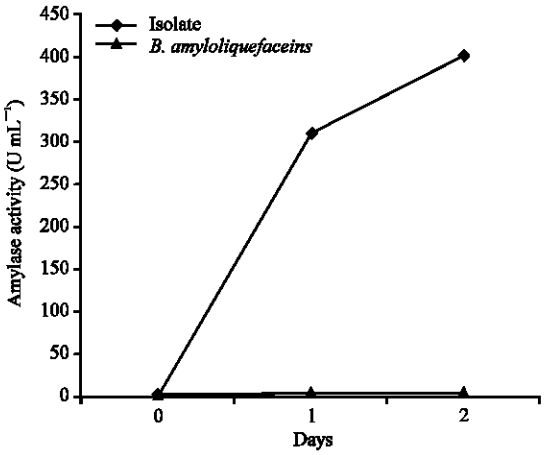

Fig. 1: The comparison of α-amylase production between *B. amyloliquefaceins* and isolate

First time course, was performed in 1 L bioreactor with fermentation medium using cassava starch under standard conditions to study the α-amylase production. Bioreactor was a good assembly for fermentation because control of parameters can be done. Highest α-amylase activity was observed in bioreactor was 400 U mL^{-1} of culture fluid (cf) on second day, with protein 310.4 µg mL^{-1} of cf. Reducing sugars showed continuous lower trend in consecutive days (Table 1).

Second time course was performed with the isolate obtained from sample. When compared the α-amylase production by isolate and standard strain *Bacillus amyloliquefaceins* it showed poor results. Alpha amylase production was very less nearly 2.9 U mL^{-1} on day third as compared to *Bacillus amyloliquefaceins* (Fig. 1 and Table 1, 2). This was the highest yield of α-amylase with isolates. Low protein and virtually no reducing sugars were found in assays during the analysis of samples of isolate.

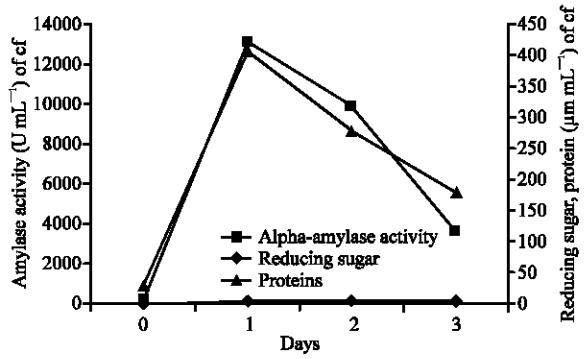

Fig. 2: Time course of *Bacillus amyloliquefaciens* grown 7% PEG-containing Cassava starch under standard conditions: temperature 37°C, inoculum size 5% (v/v), substrate concentration 1% (w/v) and aerobic conditions

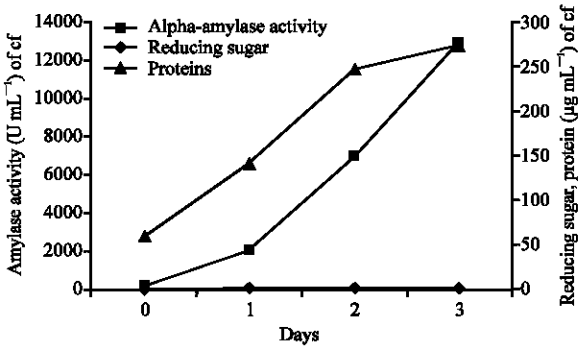

Fig. 3: Time course of *Bacillus amyloliquefaciens* grown 2% PEG-containing Cassava starch under standard conditions: temperature 37°C, inoculum size 5% (v/v), substrate concentration 1% (w/v) and aerobic conditions

We studied the effect of different concentration of polyethylene glycol (PEG) in production medium for production of α-amylase. Comparison was made among the fermentation run with and without PEG. We got promising result after adding PEG in the medium. Two concentration of PEG (4000) 2 and 7% was used in the production medium.

In the third time course, when we used 7% PEG results were different. The highest α-amylase activity observed was 13066.66 U mL^{-1} of cf (Fig. 2) with highest cell biomass 406 µg mL^{-1}. Another fact observed that the level of reducing sugar was very low in the medium with PEG. On the day one, production of α-amylase was highest than it is reduced in subsequent days (Fig. 2).

Furthermore in the last fourth time course, 2% PEG was used. Present results with medium containing 2% PEG were promising. There was gradual increase in amount of α-amylase and on third day highest activity (12933.33 U mL^{-1}) was obtained (Fig. 3). These observations was strongly supported my hypothesis that α-amylase production may be increased by using PEG in the medium. PEG solution in low concentration affect the permeability of cell membrane by affects its phospholipids layer. In fusogenic concentration of PEG, structure of cell membrane become loose and cells are more permeable to ions (Brook *et al*., 1985). It was nearly 30 times higher α-amylase production than the medium where fermentation ran without PEG. In last two time courses (Time course 3 and 4) having PEG, produce much higher amylase than the previous two time

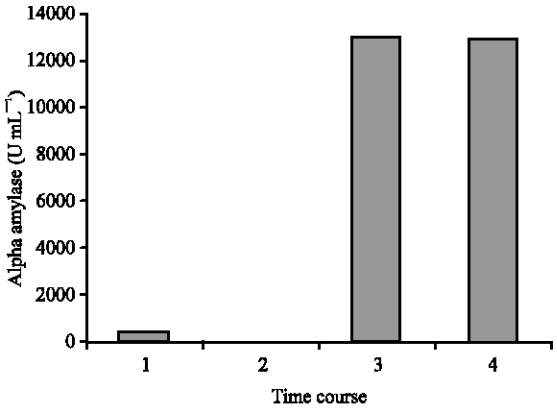

Fig. 4: Alpha amylase activity in all four time courses

courses (Time course 1 and 2) (Fig. 4). Ulger and Curakoglu (2001) also reported that supplementation of polyethylene glycols in fermentation medium for α-amylase production, indicated that 5% PEG 600 and PEG 3000 yielded 31% increase in enzyme production by *B. amyloliquefaceins*. It may be suggested that adding of PEG in the medium have some boosting effect on α-amylase production. It had been studied that the effect of PEG on the secretion of amylase by cells. PEG (600) when added in medium increase the enzyme release in *B. subtilis*. PEG has also affect on the protoplast of plant cell. Hahn-Hagerdal and coworkers found that plant protoplast permeability was increased by PEG when subjecting to the electric field (Ulger and Curakoglu, 2001). Although, some result in 7% PEG are not positive. Because some times the higher concentration of PEG precipitate the protein. In spite of this fact enhancing effect of PEG on the production can not be denied.

It was observed that the presence of PEG 200 in the dialysis membrane increased the membrane performance. It is shown by the high urea clearance and high urea permeability.

Moreover, over all results suggested that the lower concentration of PEG is far better for α-amylase production. In the present study PEG 2% had more better and uniform results in terms of α-amylase production as compared to PEG 7% (Fig. 2, 3). Present finding was very similar to the previous findings (Ulger and Curakoglu, 2001). They found that PEG (4000) 2% medium showed α-amylase activity 7.84 U mL^{-1} while 7% PEG (4000) showed α-amylase activity 6.43 U mL. In addition, the amount of PEG also affects dialysis membrane performance. High amounts of PEG greater than 10% do not improve the membranes performances (Idris and Yet, 2006).

Ultra filtration of culture sample was carried out and in which highest concentration of α-amylase (2690 U mL^{-1} of cf) was found in sample concentrate (Fig. 5), which is nearly ten times higher than the original sample (260 U mL^{-1}). Theoretically ultra filtrate should not have any amylase activity but in our result it was 89 U mL^{-1}. It might be due to leakage or damage in membrane which does not allow more than 10000 Dalton molecules to go out.

The polyethylene glycol (PEG)/salt system are suitable for downstream processing of amylase, due to its high capacity biocompatibility and low costs (Bezerra *et al*., 2006). In order to perform large-scale purification of enzymes and proteins an efficient downstream processing technique amenable to preserve biological activity is needed (Tanuja *et al*., 1997). Both Solid Substrate Fermentation (SSF) and submerged fermentation (SmF) could be used for the production of amylases, although traditionally these have been obtained from submerged cultures because of

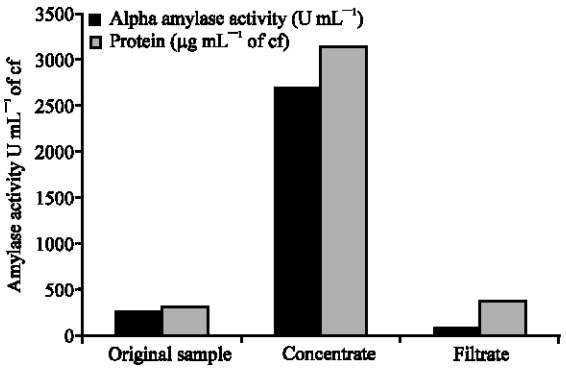

Fig. 5: Results of ultrafiltration of broth from *B. amylofacience* grown on Cassava starch in 1 L bioreactor

ease of handling and greater control of environmental factors such as temperature and pH. Various physical and chemical factors have been known to affect the production of a-amylase such as temperature, pH, period of incubation, carbon sources acting as inducers, surfactants, nitrogen sources, phosphate, different metal ions, moisture, agitation etc.

Present study suggests that *B. amyloliquefaceins* is the best bacterial strain to produce maximum quantity of α-amylase in the medium containing PEG 2%.

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